



## Genetic Effects of Captive Breeding Cause a Rapid, Cumulative Fitness Decline in the Wild

Hitoshi Araki, *et al.*

*Science* **318**, 100 (2007);

DOI: 10.1126/science.1145621

**The following resources related to this article are available online at [www.sciencemag.org](http://www.sciencemag.org) (this information is current as of October 15, 2008 ):**

**Updated information and services**, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/cgi/content/full/318/5847/100>

**Supporting Online Material** can be found at:

<http://www.sciencemag.org/cgi/content/full/318/5847/100/DC1>

This article **cites 16 articles**, 2 of which can be accessed for free:

<http://www.sciencemag.org/cgi/content/full/318/5847/100#otherarticles>

This article has been **cited by** 9 article(s) on the ISI Web of Science.

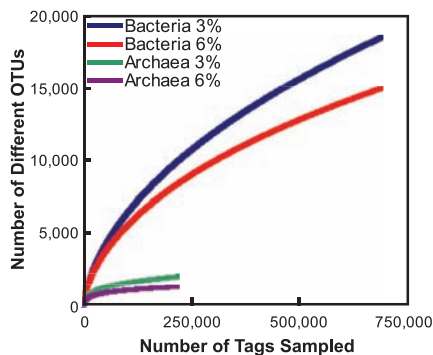
This article appears in the following **subject collections**:

Genetics

<http://www.sciencemag.org/cgi/collection/genetics>

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:

<http://www.sciencemag.org/about/permissions.dtl>



**Fig. 2.** Rarefaction curves for total bacterial and archaeal communities at the two sampling sites F5312 and F5396 at 3% and 6% difference levels.

lications for our ability to sample and identify all the ecologically relevant members of microbial communities in other high-diversity habitats, such as soils (22), microbial mats (23), and communities where low-abundance taxa may play crucial roles, such as the human microbiome. It provides a comparative population structure analysis with statistically significant descriptions of diversity and relative abundance of microbial populations. These large estimates of phylogenetic diversity at every taxonomic level present a challenge to large-scale microbial community genomic surveys. Metagenomic studies seek to inventory the full range of metabolic capabilities that define ecosystem function or to determine their context within assembled genomic scaffolds. Our results suggest that even the largest of published metagenomic investigations inadequately represent the full extent of microbial diversity, as they survey only the most highly abundant taxa (11).

In addition, the importance of microdiversity cannot be overlooked, and metagenomic community reconstructions from the two vents studied here would likely be largely chimeric assemblies of sequences from closely related phylotypes, which may mask important biological differences. Methods such as the massively parallel tag sequencing approach used here, combined with the multitude of other quantitative and descriptive tools now available to microbial ecologists, can serve as necessary accompaniments to metagenomic gene surveys as we strive to understand the impact of diversity on ecosystem function and long-term stability (24).

#### References and Notes

1. S. J. Giovannoni, T. B. Britschig, C. L. Moyer, K. G. Field, *Nature* **345**, 60 (1990).
2. D. B. Rusch *et al.*, *PLoS Biol.* **5**, e77 (2007).
3. S. Yooseph *et al.*, *PLoS Biol.* **5**, e16 (2007).
4. M. L. Sogin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12115 (2006).
5. H. P. Johnson, R. W. Embley, *J. Geophys. Res.* **95**, 12689 (1990).
6. R. W. Embley, E. T. Baker, *Eos* **80**, 213 (1999).
7. J. A. Huber, D. A. Butterfield, J. A. Baross, *Appl. Environ. Microbiol.* **68**, 1585 (2002).
8. J. A. Huber, D. A. Butterfield, J. A. Baross, *FEMS Microbiol. Ecol.* **43**, 393 (2003).
9. D. A. Butterfield *et al.*, in *The Subseafloor Biosphere at Mid-Ocean Ridges*, W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross, S. C. Cary, Eds. (American Geophysical Union, Washington, DC, 2004), pp. 269–289.
10. A. Maruyama, T. Urabe, J. Ishibashi, R. A. Feely, E. T. Baker, *Cah. Biol. Mar.* **39**, 249 (1998).
11. See supporting material on *Science Online*.
12. P. D. Schloss, J. Handelsman, *Appl. Environ. Microbiol.* **71**, 1501 (2005).
13. A. Chao, *Scand. J. Stat.* **11**, 265 (1984).
14. B. J. Campbell, A. S. Engel, M. L. Porter, K. Takai, *Nat. Rev. Microbiol.* **4**, 458 (2006).

15. L. R. Moore, G. Rocap, S. W. Chisholm, *Nature* **393**, 464 (1998).
16. S. G. Acinas *et al.*, *Nature* **430**, 551 (2004).
17. G. Rocap, D. L. Distel, J. B. Waterbury, S. W. Chisholm, *Appl. Environ. Microbiol.* **68**, 1180 (2002).
18. D. M. Raup, *Paleobiology* **1**, 333 (1975).
19. R. Massana, E. F. DeLong, C. Pedros-Alio, *Appl. Environ. Microbiol.* **66**, 1777 (2000).
20. S. M. Huse, J. A. Huber, H. G. Morrison, M. L. Sogin, D. Mark Welch, *Genome Biol.* **8**, R143 (2007).
21. C. Pedros-Alio, *Trends Microbiol.* **14**, 257 (2006).
22. P. D. Schloss, J. Handelsman, *PLoS Comput. Biol.* **2**, e92 (2006).
23. R. E. Ley *et al.*, *Appl. Environ. Microbiol.* **72**, 3685 (2006).
24. E. F. DeLong, *Nat. Rev. Microbiol.* **5**, 326 (2007).
25. We thank the NOAA Pacific Marine Environmental Laboratory Vents Program, the ROPOS Remotely Operated Vehicle, and S. Bolton for field support, and P. Schloss and L. Amaral Zettler for assistance in data analysis and primer design. Supported by NASA Astrobiology Institute Cooperative Agreement NNA04CC04A (M.L.S.), a National Research Council Research Associateship Award and L'Oréal USA Fellowship (J.A.H.), the Alfred P. Sloan Foundation's ICOMM field project, the W. M. Keck Foundation, and the Joint Institute for the Study of the Atmosphere and Ocean under NOAA Cooperative Agreement NA17RJ1232, Contribution 1388. This is NOAA Pacific Marine Environmental Laboratory Contribution 3047. The new sequences reported in this paper have been deposited in the NCBI Short Read Archive under accession numbers SRA000195 and SRA000196. The zip file available for download via [http://jbpc.mbl.edu/research\\_supplements/g454/20070822-private/supplemental.zip](http://jbpc.mbl.edu/research_supplements/g454/20070822-private/supplemental.zip) contains all the fasta-formatted trimmed reads used in the analyses.

#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/318/5847/97/DC1](http://www.sciencemag.org/cgi/content/full/318/5847/97/DC1)  
Materials and Methods  
SOM Text  
Figs. S1 and S2  
References

19 June 2007; accepted 28 August 2007  
10.1126/science.1146689

## Genetic Effects of Captive Breeding Cause a Rapid, Cumulative Fitness Decline in the Wild

Hitoshi Araki,\* Becky Cooper, Michael S. Blouin

Captive breeding is used to supplement populations of many species that are declining in the wild. The suitability of and long-term species survival from such programs remain largely untested, however. We measured lifetime reproductive success of the first two generations of steelhead trout that were reared in captivity and bred in the wild after they were released. By reconstructing a three-generation pedigree with microsatellite markers, we show that genetic effects of domestication reduce subsequent reproductive capabilities by ~40% per captive-reared generation when fish are moved to natural environments. These results suggest that even a few generations of domestication may have negative effects on natural reproduction in the wild and that the repeated use of captive-reared parents to supplement wild populations should be carefully reconsidered.

Captive breeding was originally used as a form of conservation for the most critically endangered species, but is now widely used for the restoration of declining natural populations (1–3). In theory, captive-reared organisms may accumulate deleterious alleles that could hinder the recovery of natural popula-

tions (3–6). However, the extent to which captive-reared individuals contribute genetically to the restoration of natural populations is not known.

Hatchery programs for enhancing threatened populations of Pacific salmon and steelhead trout (*Oncorhynchus* spp.) release more than five billion juvenile hatchery fish into the North

Pacific every year (7, 8). Although most of these hatchery programs are meant to produce fish for harvest, an increasing number of captive breeding programs are releasing fish to restore declining natural populations (8, 9). Hatchery fish breed in the wild, and many natural populations are affected by hatchery fish. The use of hatchery-reared fish as broodstock (parents of hatchery fish) for many generations has resulted in individuals that contribute less to the gene pool (are less fit), in comparison with wild fish, in natural environments (10–12). On the other hand, captive breeding programs that use local wild fish as broodstock are expected to produce hatchery fish having minimal differences in fitness from wild fish. Nevertheless, such captive-reared fish can be genetically distinct from wild fish for a variety of traits (13–16). Thus, it is a real concern that these fish will also have low fitness (reproductive success) in natural environments.

A two-generation pedigree of DNA-based parentage analyses of steelhead (*Oncorhynchus*

Department of Zoology, 3029 Cordley Hall, Oregon State University Corvallis, OR 97331, USA.

\*To whom correspondence should be addressed. E-mail: arakih@science.oregonstate.edu

*mykiss*) in the Hood River in Oregon (U.S.A.) showed that the first generation of captive-reared fish had natural reproductive success indistinguishable from that of wild fish in two out of three run-years (17). (Each run-year begins when parents arrive at the river to spawn.) This comparison, however, neglected the fact that captive-reared and wild individuals experience different environments as juveniles, which might affect mating behaviors, fecundity, and/or fertility (18). Therefore, it is difficult to disentangle environmental effects from genetic effects of a difference or lack of difference in reproductive success (17).

In this study, we investigated the strength of genetic effects of domestication on the reproductive success of captive-reared individuals in the wild. Confounding environmental effects were avoided by comparing captive-reared individuals with different histories of captive breeding in the previous generation (Fig. 1). We reconstructed a three-generation pedigree of the winter-run steelhead in the Hood River (19) and compared adult-to-adult reproductive success (number of wild-born, adult offspring per parent) of two types of captive-reared fish (designated C): captive-reared fish from two wild-born parents (C[WxW]), and captive-reared fish from a wild-born parent and a first-generation captive-reared parent (C[CxW]). C[CxW] and C[WxW] were born in the same year, reared in the same hatchery without distinction, and released at the same time. Both fish originated from the same local population, so we can also exclude the in-

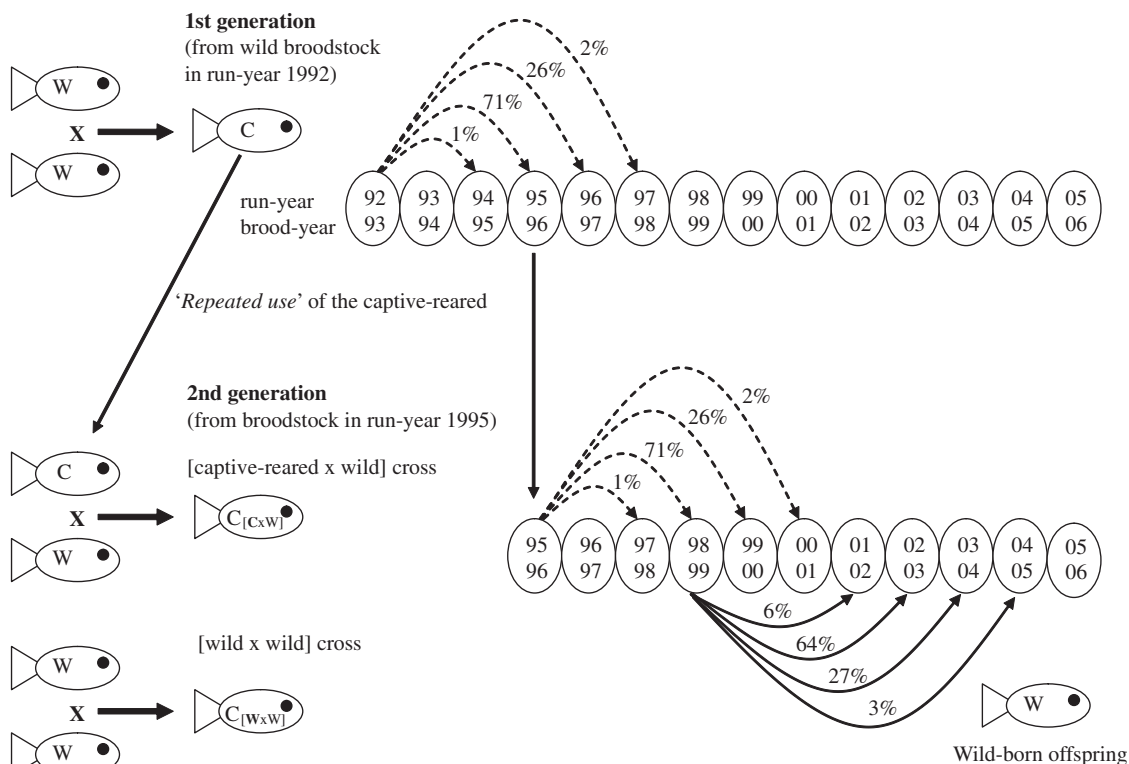
fluence of local origin. The only difference between them is half of the genome. The half genome in C[CxW] was inherited from the captive-reared parent and experienced captivity for two consecutive generations (during the egg-to-juvenile development). The other half in C[CxW] was from the wild parent and experienced captivity for one generation (C[CxW] itself). In contrast, the entire genome of the C[WxW] experienced captivity for one generation. Thus, by comparing C[CxW] with C[WxW], we were able to evaluate the effect of a single extra generation of captive rearing on subsequent reproductive success in the wild, while controlling for the effect of rearing environment (Fig. 1).

We estimated the reproductive success of 547 C[CxW] and 193 C[WxW] over three run-years (1998–2000) (19). On the basis of the parentage analysis, we assigned 355 wild-born, returning adult offspring to at least one of their C[CxW] or C[WxW] parents (Table 1). Our estimate of relative reproductive success (RRS) with an unbiased method (20) revealed that the overall reproductive success of C[CxW] is only 55% that of C[WxW] ( $P = 0.009$  by one-tailed permutation tests). We also compared the reproductive success of C[CxW] and C[WxW] from single cohorts (i.e., using only 3-year-olds at the time of spawning) (Table 1). In this comparison, environmental differences were eliminated because both types of hatchery fish were born, returned, and spawned in the same environments in the same year. The smaller sample size re-

sulted in lower power, but the overall estimate was very similar to the above result (single-cohort RRS of C[CxW] to C[WxW] = 0.609,  $P = 0.042$ ).

In addition to comparing reproductive success between C[WxW] and C[CxW], we also compared the reproductive success of these captive-reared fish to that of wild-born fish (W) returning in the same run-years (1998–2000). Overall RRS of C[WxW] to W was 0.595 and that of C[CxW] to W was 0.310 [both  $P < 0.001$ , (table S1)]. Our estimates of RRS for C[WxW] can be compared with those from our previous study of run years 1995–1997 (17) (table S1). Interestingly, the estimate from run years 1998–2000 was significantly lower than the average RRS  $\sim 1$  estimated from run-years 1995–1997 (17) (Fig. 2A). One possible explanation for this difference is presence of C[CxW] on the spawning grounds in 1998–2000. For example, reproductive interaction between C[CxW] and C[WxW] might reduce the average reproductive success of C[WxW] if C[WxW] tend to mate more with C[CxW] than with W. Another possibility is nonadditive fitness effects such that mating between hatchery fish results in lower fitness than expected. In our data, nonrandom mating was supported by a test of independence [ $P < 0.001$  for all three run-years (table S2)]. However, an excess of observed mating was found between wild parents, not between captive-reared parents. This might indicate both nonrandom mating (WxW and CxC mating preferences) and nonadditive fitness effects (i.e.,

**Fig. 1.** Distribution of run-years in which captive-reared fish and their wild-born offspring returned. Numbers in a circle represent a run-year of parents (top) and a brood-year of their offspring (bottom). The percentage on each arrow represents the proportion of adults that return in each subsequent year, which differs between captive-reared fish (dotted line) and wild fish (solid line). C[CxW] were iteratively created from wild individuals and the first generation of captive-reared individuals that returned in run-year 1995; subsequent C[CxW] individuals were created from those individuals returning in 1996 and so forth. These first-year C[CxW] fish returned to spawn mostly in run-year 1998, and we estimated their reproductive success by matching them to the wild-born offspring that returned in run-year 2001–2004.



low fitness of CxC), although analyses of reproductive success between crosses did not show the presence of nonadditive genetic effects {RRS of

$[C[CxW] \times C[WxW]]$  to  $[W \times C[WxW]] = 1.1$  in run-year 2000,  $P = 0.878$  (table S3)}. Over six run-years of data (1995–2000), four of six years

showed lower fitness of C[WxW] (overall RRS of C[WxW] to  $W = 0.848$ ,  $P < 0.001$ ).

**Table 1.** RRS (relative number of adult offspring per parent) of two types of captive-reared fish,  $C[CxW]$  versus  $C[WxW]$ . RRS is given as an unbiased estimate (19, 20).  $P$  values were calculated by a one-tailed permutation test. Statistical power represents the minimum effect size (displayed as RRS) detectable with 80% and 95% power. [See (19) and footnote of table S1 for details.] When all parents were compared, overall RRS was estimated using weighted geometric means. The  $P$  values were calculated on the basis of Fisher's combined probability (19). For single cohorts, only 3-year-old C[CxW] and C[WxW] were compared. \* $P < 0.05$ , \*\* $P < 0.01$

Run-year	N [offspring assigned]	RRS	P value	Statistical power (80%/95%)
<i>From all parents</i>				
[Male]				
1998	79	0.341	0.035*	0.626/0.341
1999	26	0.577	0.239	0.462/0.296
2000	79	0.856	0.381	0.701/0.540
Overall male		0.545	0.074	
[Female]				
1998	74	0.504	0.238	0.384/0.156
1999	25	0.212	0.007**	0.468/0.321
2000	72	0.828	0.319	0.706/0.567
Overall female		0.547	0.020*	
Overall both sexes		0.546	0.009**	
<i>From single cohorts</i>				
[Male]				
1998	48	0.361	0.044*	0.582/0.361
1999	25	0.502	0.171	0.502/0.324
2000	77	0.862	0.390	0.705/0.543
Overall male		0.596	0.070	
[Female]				
1998	22	0.985	0.591	0.257/0.075
1999	15	0.137	0.036*	0.351/0.137
2000	56	0.798	0.319	0.641/0.480
Overall female		0.631	0.125	
Overall both sexes		0.609	0.042*	

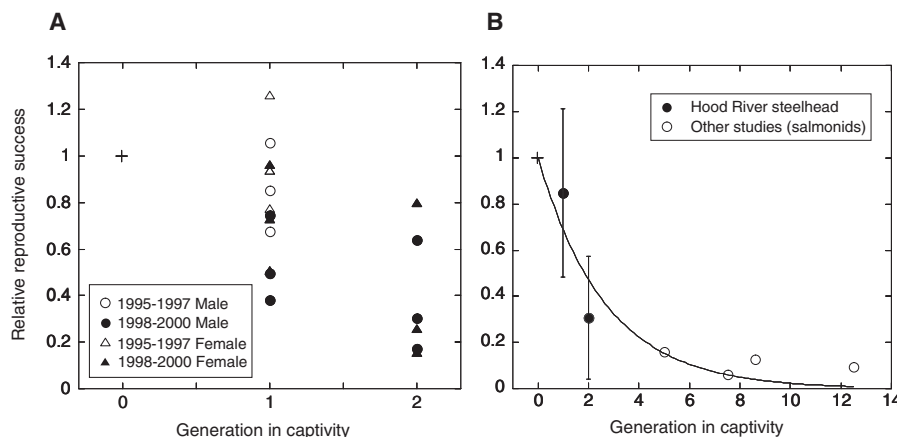
One factor we cannot completely exclude in these comparisons is nongenetic grandparental effects, which have been demonstrated in various organisms, including fish (21–24). However, known grandparental effects are mostly female-specific (i.e., grandmaternal egg effects). The reproductive success of C[CxW] did not depend on the sex of the captive-reared parent (overall RRS of C[CxW] with a captive-reared mother to C[CxW] with a captive-reared father = 1.009,  $P = 0.81$ ). Similarly, there were no noticeable maternal effects on the reproductive success when hatchery and wild fish mated in the wild, either in this study or in our previous study [i.e., number of resulting offspring did not depend on which type of fish was the mother (table S1) (17)]. Thus, the grandparental effect is less likely in this case, and the most likely explanation for the fitness decline is a genetic disadvantage of C[CxW] resulting from the half genome exposed to artificial environments for an additional generation.

Our data suggest a sharp decline in reproductive success follows a very short time in captivity (Fig. 2A). We also conducted a meta-analysis to compare our data with those available for four hatchery stocks for which we know the number of generations in hatcheries (19, 25). These data fit very well on an exponentially declining curve (Fig. 2B), despite the fact that the previous data include RRS estimates using different species and methods and that they are subject to confounding environmental effects (19, 25). It shows 37.5% fitness decline per captive-reared generation, suggesting that the fitness decline of captive-reared fish can be remarkably fast. Because any purely environmental effects should not accumulate over time, the continued decline with generations in captivity (Fig. 2) further supports genetic effects as the cause.

The evolutionary mechanism causing the fitness decline remains unknown. We suspect that unintentional domestication selection and relaxation of natural selection, due to artificially modified and well-protected rearing environments for hatchery fish, are probably occurring (SOM text). Considering the mating scheme for C[CxW] and the generation time for the fitness decline, however, inbreeding depression and accumulation of new mutations should not affect these results. Regardless, our data demonstrate how strong the effects can be and how quickly they accumulate. To supplement declining wild populations, therefore, repeat use of captive-reared organisms for reproduction of captive-reared progenies should be carefully reconsidered.

**References and Notes**

1. M. L. Cuenco, T. W. H. Barkman, P. R. Mundy, in *Genetic Conservation of Salmonid Fishes*, J. G. Cloud, G. H. Thorgaard, Eds. (Plenum Press, New York, 1993), pp. 269–294.



**Fig. 2.** (A) Estimated RRS of captive-reared fish relative to wild fish, plotted against generation time in captivity. Each point represents an estimate from a run-year and sex. The point at generation 0 represents wild fish as a control (marked as a cross). Estimates of the RRS of C[WxW] are plotted at generation 1 and C[CxW] at generation 2. Three years of data at generation 1 (open plots) are from (17). (B) Meta-analysis of the RRS of captive-reared versus wild fish plotted against generation time in captivity of other salmonid species. Solid circles are the estimates from our data (weighted geometric means from Fig. 2A). The bar represents 1 SD. The other four points are from two studies on steelhead, one on brown trout, and one on Atlantic salmon (table S4) from (25). The exponential regressions were obtained as  $y = e^{-0.375x}$  (correlation coefficient = 0.962), which suggest that fitness in the wild is reduced 37.5% per generation of captive breeding.

2. P. J. S. Olney, G. M. Mace, A. Feistner, *Creative Conservation: Interactive Management of Wild and Captive Animals* (Chapman & Hall, London, ed. 1, 1994).
3. R. Frankham, D. A. Briscoe, J. D. Ballou, *Introduction to Conservation Genetics* (Cambridge Univ. Press, Cambridge, 2002).
4. M. Lynch, M. O' Hely, *Conserv. Genet.* **2**, 363 (2001).
5. M. J. Ford, *Conserv. Biol.* **16**, 815 (2002).
6. D. Goodman, *Can. J. Fish. Aquat. Sci.* **62**, 374 (2005).
7. W. R. Heard, "An estimate of total 1992 hatchery releases of the North Pacific Ocean and adjacent seas." [North Pacific Anadromous Fish Commission Doc. 154, Auke Bay Fisheries Laboratory, Alaska Fisheries Science Center, National Marine Fisheries Service (NMFS), National Oceanic and Atmospheric Administration (NOAA), Juneau, AK, 1995].
8. X. Augerot, D. N. Foley, *Atlas of Pacific Salmon: The First Map-Based Status Assessment of Salmon in the North Pacific* (Univ. of California Press, Berkeley, CA, 2005).
9. R. N. Williams, *Return to the River: Restoring Salmon to the Columbia River* (Elsevier Academic Press, Amsterdam, 2006).
10. I. A. Fleming et al., *Proc. R. Soc. London Ser. B* **267**, 1517 (2000).
11. I. A. Fleming, E. Peterson, *Nord. J. Freshw. Res.* **75**, 71 (2001).
12. B. Berejikian, M. J. Ford, "Review of relative fitness of hatchery and natural salmon." (NMFS-NWFSC-61, Northwest Fisheries Science Center, Seattle, WA, 2004).
13. R. R. Reisenbichler, J. D. McIntyre, *Can. J. Fish. Res. Board* **34**, 123 (1977).
14. D. D. Heath, J. W. Heath, C. A. Bryden, R. M. Johnson, C. W. Fox, *Science* **299**, 1738 (2003).
15. T. N. Pearsons, A. L. Fritts, J. L. Scott, *Can. J. Fish. Aquat. Sci.* **64**, 803 (2007).
16. A. L. Fritts, J. L. Scott, T. N. Pearsons, *Can. J. Fish. Aquat. Sci.* **64**, 813 (2007).
17. H. Araki, W. R. Ardren, E. Olsen, B. Cooper, M. S. Blouin, *Conserv. Biol.* **21**, 181 (2007).
18. B. A. Berejikian, E. P. Tezak, S. L. Schroder, C. M. Knudsen, J. J. Hard, *ICES J. Mar. Sci.* **54**, 1040 (1997).
19. Methods are available as supporting material on Science Online.
20. H. Araki, M. S. Blouin, *Mol. Ecol.* **14**, 4097 (2005).
21. M. J. Hercus, A. A. Hoffmann, *Proc. R. Soc. London Ser. B* **267**, 2105 (2000).
22. A. Kyne, S. Toft, *Ecol. Entomol.* **31**, 322 (2006).
23. D. Reznick, *Evolution Int. J. Org. Evolution* **35**, 941 (1981).
24. J. Lindström, *Trends Ecol. Evol.* **14**, 343 (1999).
25. "Report for the meeting held August 20 to September 2, 2004" (Salmon Recovery Science Review Panel, Northwest Fisheries Science Center, Seattle, 2004).
26. We thank R. S. Waples, M. Ford, and four anonymous reviewers for helpful discussions, W. R. Ardren, C. Criscione, R. VanDam and the Center for Genome Research and Biocomputing in Oregon State University for help with laboratory work and E. Olsen, R. French, J. Gidley, and staff of the Oregon Department of Fish and Wildlife (ODFW) for technical help and advice. This research was funded by contracts to M.S.B. from the Bonneville Power Administration and the ODFW.

#### Supporting Online Material

www.sciencemag.org/cgi/content/full/318/5847/100/DC1  
Methods  
SOM Text  
Tables S1 to S5  
Appendix  
References

24 May 2007; accepted 21 August 2007  
10.1126/science.1145621

## Glia Promote Local Synaptogenesis Through UNC-6 (Netrin) Signaling in *C. elegans*

Daniel A. Colón-Ramos,<sup>1</sup> Milica A. Margeta,<sup>1,2</sup> Kang Shen<sup>1,2\*</sup>

Neural circuits are assembled through the coordinated innervation of pre- and postsynaptic partners. We show that connectivity between two interneurons, AIY and RIA, in *Caenorhabditis elegans* is orchestrated by a pair of glial cells that express UNC-6 (netrin). In the postsynaptic neuron RIA, the netrin receptor UNC-40 (DCC, deleted in colorectal cancer) plays a conventional guidance role, directing outgrowth of the RIA process ventrally toward the glia. In the presynaptic neuron AIY, UNC-40 (DCC) plays an unexpected and previously uncharacterized role: It cell-autonomously promotes assembly of presynaptic terminals in the immediate vicinity of the glial cell endfeet. These results indicate that netrin can be used both for guidance and local synaptogenesis and suggest that glial cells can function as guideposts during the assembly of neural circuits in vivo.

Neural circuit formation requires an intricate orchestration of multiple developmental events, including cell migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis (1–3). These developmental events are coordinated in pre- and postsynaptic neuronal partners to form the functional neural circuits that underlie behaviors. Although the organization and specificity of these neural circuits is well documented, the cellular and molecular mechanisms that underlie their precise development are not well understood.

To explore how precise neural connectivity is achieved, we studied the synaptic connections between two interneurons in the *C. elegans* brain: presynaptic AIY and postsynaptic RIA. These two interneurons navigate complex cellular envi-

ronments, discriminating among multiple potential targets before finding and innervating each other at a discrete region of their respective processes (4). We generated single-cell fluorescent markers to visualize AIY-RIA connectivity in vivo and observed a discrete clustering of presynaptic AIY markers in a segment of the process we termed zone 2. This zone appears to be the specialized presynaptic region where AIY forms synapses onto RIA, as well as RIB and AIZ neurons. First, the fluorescently labeled presynaptic proteins RAB-3, ELKS-1, and SYD-2 are all more concentrated in zone 2 than in other regions of the axon (Figs. 1A and 2B and fig. S4A). Second, these markers cluster at the exact location at which AIY to RIA synapses are seen in electron micrographs of wild-type animals (fig. S1M) (5). Third, this region has a wider diameter than other regions of the axon, a property that we found to be uniquely associated with the presynaptic region of AIY in electron micrographs (fig. S1, A and M to Q). These combined properties were taken as evidence of presynaptic

differentiation and were very reproducible across animals (Fig. 1 and fig. S1).

Reconstructions of electron microscopy (EM) micrographs (5) revealed that AIY has three distinct anatomical regions throughout its process: a segment proximal to the AIY cell body that is devoid of synapses (zone 1); the synapse-rich region where AIY forms synapses onto RIA, AIZ, and RIB just as the AIY process turns dorsally (zone 2); and a distal axon segment within the nerve ring that has four to eight small presynaptic specializations (zone 3).

To identify the molecular signals that direct this precise innervation, we performed a visual genetic screen for mutants with an abnormal synapse distribution in AIY. From this screen, we isolated the *wy81* mutation, an allele of *unc-40* (fig. S2). UNC-40 (DCC, deleted in colorectal cancer) is a transmembrane immunoglobulin superfamily protein that is a receptor for the axon guidance molecule UNC-6 (netrin) (6, 7). *unc-40* animals had no detectable axon guidance defects in AIY except for an axon truncation defect observed in 7.8% of the animals ( $n = 153$  animals; fig. S3). However, they showed a highly penetrant defect in the presynaptic specialization of AIY at zone 2: 95.3% of *unc-40(wy81)* animals displayed a severe reduction of active zone markers ELKS-1::YFP (yellow fluorescent protein) and SYD-2::GFP (green fluorescent protein) and a synaptic vesicle marker, mCherry::RAB-3, in zone 2 ( $n = 128$  animals; Fig. 2, A to K, and fig. S4). In addition, the AIY axon diameter in zone 2 failed to widen into the characteristic presynaptic varicosity seen in wild-type animals (fig. S1). By contrast, in the more-dorsal zone 3 synaptic regions, *unc-40* animals had normal or increased levels of synaptic vesicle proteins and a normal or increased diameter (Fig. 2, F to I, and fig. S1). These defects suggest a specific defect in the presynaptic differentiation of AIY in zone 2, although a detailed analysis of AIY synaptic ultrastructure and function could

<sup>1</sup>Department of Biological Sciences, Stanford University, 144 Herring Laboratories, Stanford, CA 94305–5020, USA.

<sup>2</sup>Neurosciences Program, Stanford University, Stanford, CA 94305–5020, USA.

\*To whom correspondence should be addressed. E-mail: kangshen@stanford.edu